

Original scientific paper
Originalan naučni rad
 UDK: 632.482.14
 DOI: 10.7251/AGREN1501121D

University of Banjaluka, Faculty of Agriculture

*Agro-
knowledge
Journal* **A**

Sanitary Status of Pome and Stone Fruit Collection in Gene Bank in Republic of Srpska

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Abstract

Detection of viruses presence were carried out for the 225 a of pome and stone fruit trees from the collection of the Genetic Resources Institute of University of Banja Luka, located within the Botanical Garden of the University, tested by DAS-ELISA. The pome fruit trees were analyzed on presence of the following viruses: *Apple Chlorotic Leaf Spot Virus* (ACLSV), *Apple Stem Grooving Virus* (ASGV), *Apple Stem Pitting Virus* (ASPV) and *Apple Mosaic Virus* (ApMV). The stone fruits were analyzed on presence of *Plum Pox Virus* (PPV), *Prune Dwarf Virus* (PDV) and *Prunus Necrotic Ring Spot Virus* (PNRSV). All samples were tested serologically by DAS-ELISA. In addition to this, virus negative pear and apple accessions were tested for 'Candidatus *Phytoplasma mali*' and 'Candidatus *Phytoplasma pyri*' presence using nested-PCR/RFLP analyses.

Key words: Pome and Stone Fruit Viruses, Pome Fruit Phytoplasmas, DAS-ELISA, Nested-PCR/RFLP

Introduction

The present territory of Republic of Srpska, as part of Bosnia and Herzegovina, was historically exposed to the influences of different civilizations. In Bosnia and Herzegovina, and the present territory of Republic of Srpska through spontaneous or planned hybridization and selection, introduced germplasm participated in the creation of new autochthonous varieties. However, in the absence of systematic research in the past, although this area could mark a primary gene center for some species, it is practically not mentioned in the international literature. Taking into account the diversity of agricultural conditions and the effects of various civilizations throughout history it can be concluded that the Republic of Srpska is very rich in agro-biodiversity and to be considered as a gene-center of a number of cultivated species (Đurić *et al.*, 2009). Issues of conservation and sustainable use of plant genetic resources in Republic of Srpska are main goals of Genetic Resources Institute of University of Banja Luka, who is coordinator institution for implementation of the Programme of conservation of plant genetic resources of the Republic of Srpska (Đurić *et al.*, 2012). The field collections of autochthonous fruit cultivars in Banja Luka is selected for the assessment of sanitary status of fruit trees. The collection includes 65 apples (62 accessions represented with 2 trees and 3 accessions represented with 3 trees), 43 pear (36 accessions are represented with 2 trees and 7 accessions are represented with 1 tree), 5 plum (4 accessions represented with 2 trees and 1 accession represented with 1 tree), 5 cherries (2 accessions represented with 2 tree and 3 accessions represented with 1 tree), as well as 1 apricot (1 accessions represented with 2 trees) and 1 sweet cherry accession. Since the accessions in the collection obtained by grafting with cuttings that were collected up at various locations throughout the Republic of Srpska and that in most cases were the old trees of several dozens or even hundreds of years, checking of sanitary status was the first step in determining the health status of the accessions in the collection.

During 2014, implementing the activities carried out to determine the healthy status of the fruit collection, which in the first phase included verification of the virus presence. The detection was made to verify the presence of 4 economically important pome fruit viruses: *Apple Chlorotic Leaf Spot Virus* (ACLSV), *Apple Stem Grooving Virus* (ASGV), *Apple Stem Pitting Virus* (ASPV) and *Apple Mosaic Virus* (ApMV). These viruses occur frequently in mixed infections and can cause significant yield reduction (Posnette *et al.*, 1963; Desvignes, 1999). Latent viruses are more widespread

than the others (Hadidi, et al., 2003). In the previous period, determination of the presence and distribution of pome fruit viruses was done in the territory of Bosnia and Herzegovina (Lolić et al., 2007, 2010).

Besides plant viruses, other virus-like pathogens such as phytoplasmas significantly reducing quality and quantity of fruits. The most important pome fruit phytoplasmas '*Candidatus Phytoplasma mali*' (causing apple proliferation disease, AP) and '*Candidatus Phytoplasma pyri*' (causal agent of pear decline disease, PD) are quarantine pathogens responsible for great economic losses in fruit production (Seemüller and Schneider, 2004). Their presence and distribution in Bosnia and Herzegovina were ascertained (Delić et al., 2005, Lolić et al., 2010, Radulović et al., 2014). These phytoplasmas are transmitted by psyllids (Hemiptera Psyllidae) vectors which were also identified infected with corresponding phytoplasmas in Bosnia and Herzegovinian orchards (Delić et al., 2005). In addition to the vectors, spread of the phytoplasmas occurring through infected planting material. Considering fact that certified plant propagation material should be also free from phytoplasmas, pome fruit accession which were not positive in the DAS ELISA test for the viruses were submitted for the additional analyses for the phytoplasma presence

Stone fruits are sensitive to a numerous virus diseases, and may be infected with more than 25 different diseases that are transmitted by grafting (Myrta et al., 2003). Stone fruit trees were carried out to analyze presence of 3 economically important viruses: *Plum pox virus* (PPV), *Prune Dwarf Virus* (PDV) and *Prunus Necrotic Ring Spot Virus* (PNRSV). PDV and PNRSV are transmitted by mechanical inoculation, graft, pollen and seed (Matić et al., 2008a) and ACLSV is transmitted by mechanical inoculation and grafting (Martelli et al., 2007). In the previous period, determination of the presence and distribution of the mentioned stone fruit viruses was done at the territory of Bosnia and Herzegovina (Matić et al., 2008).

Material and Methods

Virus detection

Surveys and sampling for virus detection

All activities of viruses presence detection were carried out in the orchard collection of pome and stone fruit trees of the Botanical Garden of the University of Banja Luka. Choosing the specified collection was carried out due to the greater presence and abundance of plant species for analysis,

the proximity of the facility and laboratory complex, in order to achieve the best possible accuracy of the results.

A total of 225 trees of pome and stone fruit species were analyzed. Symptoms survey and sampling of 206 pome fruit trees, of which: 127 apple trees and 79 pear trees, while symptoms survey and sampling of 19 stone fruit trees, of which 9 plums, 7 cherries, 2 apricot and 1 sour cherry were carried out in autumn 2014.

Each tree were viewed and described for presence of symptoms as well as photography. Five to 10 well developed leaves was taken and further processed in a laboratory for analysis for the presence of viruses.

ELISA test

All collected samples were tested by ELISA (Enzyme Linked Immuno Sorbent Assay). The Double Antibody Sandwich-ELISA (DAS-ELISA) (Clark and Adams, 1977) of pome fruit tree viruses ACLSV, ApMV, ASPV and ASGV, as well as serological tests of stone fruit tree viruses PPV, PDV and PNRSV were carried out with commercial kit and manufacturer's recommendation. For detection of these viruses were used DAS ELISA assay with specific antibodies to each virus, Bioreba (Switzerland).

Fully developed leaves from the orchard collection of pome and stone fruit trees were used as a material for the ELISA testing. Polystyrene microtiter plates were coated with IgG specific for each virus, diluted in coating buffer, and incubated (for 4 hours at 30°C in a humid chamber). The plates were washed 3 times for 3 minutes with washing buffer, and after that the samples were added. Samples were prepared as follows: 0.5 g of 5-8 leaves with leaf tissue grinded with extraction buffer in a ratio of 1:20. Per 200µl of the herbal extract was placed into each well, applying 2 wells for each individual sample. After samples incubation (for 2 at 30°C hours in a humid chamber), the plates were washed. Following three washes, 200µl of conjugated antibodies were added per well and plates were incubated for 2 hours at 30°C in a humid chamber. After three washes, 200µl of freshly prepared p-nitrophenylphosphate in substrate buffer (1mg/1ml) was placed in each well and left for 20-25 minutes in the dark. The plates were incubated at room temperature and photometrically measured at 405nm after one hours, two hours and overnight on the ELISA reader (Chroma Multichannel Microplate Reader). The color reaction can be determined visually, the enzyme alkaline phosphatase detect positive reactions

developing yellow color after the addition of substrate. Reactions two or more times superior to healthy control were considered positive.

Phytoplasma detection and identification

Surveys and sampling for phytoplasma detection and identification

In the first decade of October 2015, tress from pome fruit collection which were negative for the virus infection were visually inspected for the presence of phytoplasma symptoms and sampled for the laboratory analyses. Leaf and root samples were sampled from 25 pear and 40 apple trees. Samples were stored on cold until DNA extraction procedure.

Total DNA extraction

Total DNAs were extracted from the leaf midribs and root phloem scrapings from each sample. DNeasy Plant Mini Kit (Qiagen, USA) was used for the extraction procedure following protocol described by Green *et al.*, 1999.

Nested-PCR/RFLP

For phytoplasma detection and identification in the pear and apple samples nested polymerase chain reactions, nested-PCR followed by Restriction fragment length polymorphisms, RFLP were employed. After direct PCR with phytoplasma universal primers P1/P7 (Deng and Hiruki 1991; Smart *et al.*, 1996), obtained product serve us as a template for the nested PCR with f01/r01 primers (Lorenz *et al.*, 1995) specific for detection of phytoplasmas from 16SrX ribosomal group. Reactions were done in total volume of 25 µl in PCR thermocycler (Applied Biosystems 2720) applying the following thermal steps 94°C for 2 min (initial denaturation), 38 cycles 9°C for 1 min (denaturation), 55°C for 1 min (hybridization), 7°C for 2 min (extension) and final extension 10 min at 72°C. Results were visualized by electrophoresis on 1% agarose gel in 1xTAE buffer.

For identification of phytoplasma from 16SrX group fo1/ro1 positive PCR products were submitted to the RFLP digestion with *Bsa*AI and *Ssp*I endonucleases. RFLP results were observed on 3% agarose gel in 1xTBA buffer.

Results and Discussion

Results for virus detection

Pome fruit viruses are frequently latent and therefore without visible symptoms or symptoms may not be obvious. The most presence of leaf chlorosis symptoms that is associated with the presence of ACLSV (Figure 1 and 2). The second most frequent symptom in the collection orchard is vein yellows, which is associated with the presence ASPV (Posnette, 1957) (Figure 3). The symptoms of vein yellows were more pronounced in pears than in apples. Therefore the symptoms of leaf chlorosis recorded at accessions where the ELISA gave negative results for the presence of ACLSV as well as ELISA negative results for accessions with nerves yellowing, which was associated with the presence ASPV. The presence of symptoms in orchard collection for pome fruit species recorded incompatibility with results of ELISA test.



Fig. 1. Chlorotic leaves symptoms of pear cv. Lubeničarka positive for the presence of ACLSV

Simptomi hloroze listova kruške – sorte Lubeničarka, pozitivne na prisustvo ACLSV-a



Fig. 2. Chlorotic leaves symptoms of apple cv. Zveka positive for the presence of ACLSV

Simptomi hloroze listova jabuke – sorte Zveka, pozitivne na prisustvo ACLSV-a



Fig. 3. Vein yellow symptoms of pear cv. Glibanjka positive for the presence of ASPV

Simptomi žutila lisnih nerava kod kruške – sorte Glibanjka, pozitivne na prisustvo ASPV-a

In addition to the symptoms for stone fruit tree, symptoms associated with a PPV were visible, but not determined the presence of other symptoms in the orchard collection. The accessions had displayed no symptoms of viral infection although DAS-ELISA had indicate the presence of PDV. The assumption is that a longer period is necessary for the manifestation of visible symptoms.

Considering all tested apple samples (127 apple trees), DAS ELISA results showed that: 80 apple trees were infected at least with one virus, while in 47 apple trees were not viruses infected. From a total of 80 infected apple trees, 47 trees had individual infection, while 33 trees had mixed infection, confirmed the presence of two or more tested viruses. The infection were recorded at 16 accessions in pairs (32 trees), at 13 accessions were recorded individual infection (13 trees) and at 3 accessions who were represented in the collection with 1 tree were also recorded infection.

Tab. 1. The number of infected trees of pome fruit: individual and mixed infection
Broj inficiranih stabala jabučastih voćaka: pojedinačne i mješovite infekcije

	<i>APPLE</i> JABUKA	<i>PEAR</i> KRUŠKA
ACLSV	35	10
ApMV	-	-
ASPV	2	3
ASGV	10	-
<i>INDIVIDUAL INFECTION:</i> POJEDINAČNE INFEKCIJE:	47	13
ACLSV + ApMV	2	-
ACLSV + ASGV	12	2
ACLSV + ASPV	11	-
ASPV + ASGV	1	4
ACLSV + ASPV + ASGV	6	-
ACLSV + ApMV + ASGV	1	-
<i>MIXED INFECTION:</i> MJEŠOVITE INFEKCIJE:	33	6
<i>TOTAL / UKUPNO</i> <i>(individual + mixed)</i> <i>(pojedinačne + mješovite)</i>	80	19

Considering all tested pear samples (79 pear trees), DAS ELISA results showed that: 19 pear trees were infected at least with one virus, while in 60 pear trees were not viruses infected. From a total of 19 infected pear

trees, 13 trees had individual infection, while 6 trees had mixed infection, confirmed the presence of two or more tested viruses. The infection were recorded at 7 accessions in pairs (14 trees), at 5 accessions were recorded individual infection (5 trees) and at 1 accessions who were represented in the collection with 1 tree were also recorded infection.

Considering all tested stone fruit samples (19 trees), DAS ELISA results showed that: 11 trees were infected at least with one virus, while in 4 trees were not viruses infected. From a total of 11 infected stone fruit trees: 4 plum trees, 1 cherry and 1 apricot were infected by PPV while 5 cherry trees were infected by PDV. At all tested stone fruit trees, the presence of PNRSV or mixed infections were not confirmed. The infection in pairs were recorded at 2 accessions of plum (4 trees), 2 accessions of cherry (4 trees). Individual infection were recorded at 2 accessions of cherry who were represented in the collection with 1 tree. Also, individual infection was recorded at apricot (1 tree) but infection at sour cherry was not recorded.

Tab. 2. The number of infected stone fruit accessions

Broj inficiranih prinova koštičavih voćaka

STONE FRUITS <i>KOŠTIČAVE VOĆKE</i>	<i>PPV</i>	<i>PDV</i>	<i>PNRSV</i>
PLUM / <i>ŠLJIVA</i>	4 / 9	0 / 9	0 / 9
CHERRY / <i>TREŠNJA</i>	1 / 7	5 / 7	0 / 7
SOUR CHERRY / <i>VIŠNJA</i>	0 / 1	0 / 1	0 / 1
APRICOT / <i>KAJSIJA</i>	1 / 2	0 / 2	0 / 2
<i>TOTAL / UKUPNO</i>	6	5	0

DAS ELISA test for virus detection of pome and stone fruit trees could be used as particularly important as a quick, time consuming, preliminary test for sample selection for further laboratory assay.

Results for phytoplasma detection and identification

During the surveys phytoplasma-like symptoms were observed only on pear accessions while on the apple were not typical symptoms for the apple proliferation disease. On the pear accessions we recorded some of the pear decline symptoms such as leaf roll, small leaves and yellowing/reddening (Fig. 4).

Nested-PCR/RFLP analyses showed that 9/25 pear and 3/40 apple samples from accessions were infected with '*Ca. P. pyri*' and '*Ca. P. mali*', respectively.



Fig. 4. Pear decline symptoms on pear cv. Kantaruša
Simptomi pear decline-a na sorti kruške Kantaruša

Conclusion

It is necessary in the future inventarisation of the territory of the Republic of Srpska to do collecting of accessions which are not introduced in collections of the Institute. Besides increasing of the number of accessions in collections, it is very important to check their sanitary status. Accessions on which was confirmed presence of viruses and phytoplasma will be introduced to the process of thermotherapy and those in which is confirmed absence of viruses and phytoplasmas will be used for multiplication. Since the accessions conserved in the *in situ* system are old tenth and even hundreds years, the main goal is to healed accessions put back in the on farm system of conservation. In this way they will contribute to the popularization, conservation and sustainable use of genetic resources of the Republic of Srpska.

Beside collecting and raising orchard collection, for the further activities a very great importance was determination of sanitary status of pome and stone fruit trees in the Gene Bank of the Republic of Srpska at the Institute of Genetic Resources, University of Banja Luka.

The presence of symptoms in orchard collection for pome fruit species recorded incompatibility with finally results of DAS ELISA test what was expected since pome fruit viruses frequently occur latent. Also, symptoms expression was dependent on environmental conditions and fruit trees oldage, considerable delay was possible before unambiguous symptoms appear. Reliable detection of latent viruses is very important in the crops that are vegetatively propagated.

DAS ELISA test revealed the presence of viruses of pome and stone fruit trees. For apple, the total number of tested trees, the presence of single or mixed viral infection was found in 62% of apples and 47 tested trees was considered healthy. For pears, the total number of tested trees, the presence of single or mixed viral infection was found in 24% of pear, while 60 tested trees was considered healthy. Considering all tested stone fruits, individual infection of PPV or PDV found at 58% trees and 4 trees were considered healthy. It has not been established PNRSV not the mixed infections.

Finally, nested-PCR/RFLP analyses showed that 16 pear and 37 apple trees were not infected with the quarantine phytoplasmas.

However it is necessary to continue with work in order to confirm the results obtained by molecular analyzes. Further work requires the introduction of tissue culture and getting healthy specimens in the collection as well as establishment of necessary infrastructure for conservation of virus and phytoplasma free mother stock.

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Received: February 12, 2015

Accepted: April 30, 2015

Sanitarni status kolekcije jabučastih i koštičavih voćaka u Banci gena Republike Srpske

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Sažetak

Detekcija prisustva virusa je izvršena na 225 jabučasta i koštičava stabla voćaka iz kolekcije Instituta za genetičke resurse Univerziteta u Banjoj Luci koja se nalazi u okviru Botaničke bašte Univerziteta. Stabla jabučastih voćaka su testirana na prisustvo sledećih virusa: virus hlorotične lisne pjegavosti (*Apple Chlorotic Leaf Spot Virus*, ACLSV), virus brazdavosti stabla jabuke (*Apple Stem Grooving Virus*, ASGV), virus jamičavosti stabla jabuke (*Apple Stem Pitting Virus*, ASPV) i virus mozaika jabuke (*Apple Mosaic Virus*, ApMV). Stabla koštičavog voća su analizirana na prisustvo virusa šarke šljive (*Plum Pox Virus*, PPV), virusa kržljivosti šljive (*Prune Dwarf Virus*, PDV) i virus nekrotične prstenaste pjegavosti (*Prunus Necrotic Ring Spot Virus*, PNRSV). Svi uzorci su serološki testirani DAS-ELISA testom. Prinove jabuke i kruške kod kojih nije utvrđeno prisustvo virusa su testirane na prisustvo 'Candidatus *Phytoplasma mali*' i 'Candidatus *Phytoplasma pyri*' primjenom nested-PCR/RFLP analiza.

Ključne riječi: Virusi jabučastog i koštičavog voća, Fitoplazme jabučastog voća, DAS-ELISA, Nested-PCR/RFLP

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Primljeno: 12. februar 2015.
Odobreno: 30. april 2015.